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13. ABSTRACT ***(Maximum 200 Words)***

Angiogenesis plays a pivotal role in tumor growth and metastasis. VEGF, an endothelial specific mitogen and an angiogenesis inducer in vivo, is one of the most important tumor angiogenesis growth factor. KDR, a VEGF receptor, appears to be the major transducer of VEGF signals in endothelial cells. A tethered intracellular antibody ("intrabody") strategy has been used successfully for both phenotypic and functional knockouts of target molecules. In this study, we have targeted a KDR single chain antibody (scFv) p3S5 to the endoplasmic reticulum (ER) using a c-terminal endoplasmic retention signal (KDEL). We hypothesized that the tethered KDR intrabody would bind newly synthesized KDR and block its transport to the surface of endothelial cells, thereby inhibiting VEGF-induced proliferation. The tethered intrabody significantly reduced KDR expression (from 82.5 ± 12.5% to 27.9 ± 13.6%, $P < 0.005$) in successfully transfected cells. These results demonstrate the potential for using an intrabody strategy to block angiogenesis.

14. SUBJECT TERMS

breast cancer, intrabody, anti-angiogenesis, vascular endothelial growth factor (VEGF)

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L S Wheeler 7/5/01

David Sane 7/3/01

PI - Signature Date

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(5). Introduction:

Tumor angiogenesis plays an important role in breast cancer growth and metastasis. Vascular endothelial growth factor (VEGF) stimulates the proliferation of endothelial cells after binding to its receptor (VEGF-R) on cell surfaces, and is a key factor in tumor angiogenesis. **The subject and scope of this research** involves generating various expression vectors for the VEGF-intrakine/intrabody and determining the effects of VEGF-intrakine/intrabody on the VEGF-R expression on endothelial cell surfaces, and evaluating the anti-angiogenesis and anti-tumor activity of this intakine/intrabody. **The purpose of this study** is to inactivate VEGF-R in vascular endothelial cells by using this intrakine/intrabody strategy and thereby, preventing endothelial cell proliferation, which may lay the groundwork for the development of a novel approach for breast cancer therapy.

(6). Body:

Various VEGF-intrabody expression vectors have been generated by using series PCRs (Fig.1). The original plasmid that contains single chain antibody scFv p3S5 was obtained from Imclone Systems Incorporated (New York, NY). The scFv p3S5 cDNA was amplified by two PCRs with the sense primers (P1:5'-GCTCCCAGATGGG TCCTGTCCCAGGTGAAACTGCAGGAGT CA-3' and P2:5'-TTTGAATTCATGGAA CATCTGTGGTTCTTCCTTCT CCTGGTGG CAGCTCCCAGATG GGTCC TGT CC-3'), and antisense primer (5'-TTTCTAGAGGATCC TTAC GCCCGTTTTATTTCCAA-3'). An HA tag sequence (YPYDVPDYA) was linked to the scFv gene by a PCR reaction with the sense primer (P1 and P2), and antisense primer (5'-TTTTCTAGAGGATCCTTAAGCATAATCTGGAACATCATATGGATACGCCCGTT TTATTTCCAA-3'). The scFv p3S5 gene tagged with HA was then linked with an ER retention signal (SEKDEL) by a PCR reaction with sense primer (P2) and antisense primer (5'-TTTTCTAGAGGATCCTTACAGCTCGTCCTTCTCGCTAG CATAATCTGGAACATCATA-3'). These DNA fragments were digested with BamHI and EcoRI, and cloned into the expression vector pIRES2-EGFP (Clontech). All of the constructs were identified by restriction enzyme digestion and confirmed by DNA sequencing (Sequencing Core Facility of Wake Forest University School of Medicine).

In order to determine the expression and intracellular localization of these intrabody vectors, these expression vectors were transfected into human umbilical vein endothelial cells using Lipofectamine reagent (Gibco BRL). The procedure followed was "Gibco transient or stable transfection of adherent cells protocol". In brief, in a 35 mm tissue culture plate, 3 X 10^5 HUVECs were seeded and incubated at 37°C, 5% CO2 incubator overnight. 2 μg of plasmid DNA and 7 μl of lipofectamine reagent were diluted into 100ul OPTI-MEM I Reduced Serum Medium (Gibco BRL), respectively. The two solutions were mixed and incubated at room temperature for 30 minutes. Following incubation, 0.8ml OPTI-MEM I Reduced Serum Medium was added to the mixture. Then, the final solution was added to the 35 mm HUVECs plate and incubated at 37°C, 5% CO2 in an incubator for 5 hours. EGM-2 complete medium (0.8 ml) was added at the

last hour of incubation. Following incubation, fresh EGM-2 medium was added to the HUVECs and the cells were incubated in a 37°C, 5% CO2 incubator. In order to determine the localization of the modified p3S5, immunofluorescent staining was performed using an antibody to the HA tag. Figure 2 demonstrates that cytoplasmic ER staining pattern was observed in the p3S5-HAK transfected HUVECs. The expression of EGFP could be detected in transfected cells using fluorescence microscopy (Figure 2)... Cells that did not express EGFP had no evidence of immunofluorescence with anti-HA (Fig 2), demonstrating specificity of the antibody and the lack of secretion of the intrabody with rebinding to neighboring cells. Cells transfected with the vector expressed EGFP, but did not exhibit immunofluorescence with anti-HA (Fig 2).

To examine the expression of modified p3S5-intrabody further, HUVECs were transfected with either pIRES2-EGFP control or p3S5-HAK and 48 hours later, the cell lysates and concentrated culture medium were immunoblotted with an anti-HA antibody. A 30 kDa protein band corresponding to p3S5 was found exclusively in the cell lysate (Fig 3, lane 3) and not in the culture medium of p3S5-HAK transfected cells (Fig. 3, lane 4). As expected, no immunoreactivity was found in control vector transfected cell lysates (Fig. 3, lane 1) or culture medium (Fig 3, lane 2).

To determine the effects of the modified p3S5-intrabody on KDR expression, HUVECs were transfected with pIRES2-EGFP (control vector), p3S5-HA, or p3S5-HAK, and 48 hour later, the cell surface expression of KDR was examined using flow cytometry (Figure 4). Two-color analysis was performed, with the results summarized in Table 1. Whereas 82.5 $\pm$ 12.5% of cells transfected with pIRES2-EGFP expressed KDR, only 27.9 $\pm$ 13.6% of cells transfected with p3S5-HAK expressed KDR ($p<0.01$). Transfection with the p3S5-HA vector without the KDEL tag was not effective in suppressing KDR expression with 78.6 $\pm$ 10.7% of the EGFP-expressing cells having detectable KDR ($p>0.1$). Even when all cells (GFP+ and GFP-) were included in the analysis, p3S5-HAK significantly reduced KDR expression ($p<0.01$).

To examine the effects of the modified p3S5-intrabody on HUVECs proliferation, HUVECs were transfected with p3S5-HAK or pIRES2-EGFP control. After 48 hours in culture, they were sorted based on EGFP expression into transfected and non-transfected groups, then treated with $VEGF_{165}$ at 15 ng/ml for 30 hours. A [^{3}H] thymidine incorporation assay was performed on these cells. The proliferation rate of the cells that had been transfected with p3S5-HAK was significantly lower than those from the same experiment that were not-transfected ($p<0.005$) (Fig. 5). Furthermore, there was no significant difference between non-transfected cells in the experimental group and the control vector groups. Thus, only the cells that expressed the p3S5-HAK construct had a significantly reduced response to $VEGF_{165}$.

(7). Key Research Accomplishments:

- Various intrabody expression vectors have been generated and expressed by HUVECs.
- Intrabody significantly decreased HUVECs surface expression of KDR, a VEGF receptor.
- Intrabody significantly inhibited HUVECs proliferation rate.

(8). Reportable Outcomes:

Presentation:
Journal Club, Cancer Biology Department of Wake Forest University School of Medicine: 03/2001.

Poster:
4th Annual American Society of Gene Therapy Meeting, Seattle, WA: 05/2001.

Paper submitted:
Blood: 04/2001.

(9). Conclusions:

Various VEGF-intrabody expression vectors have been generated and successfully transfected into, and expressed by HUVECs. The intrabody has significantly decreased HUVECs surface expression of VEGF receptor, KDR. And they also significantly inhibited HUVECs proliferation rate in vitro. Further studies will be directed to investigate the mechanisms of the intrabody as well as some antitumor effects of intrabody in vivo.

(10). References:

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(4). Lewis MJ, Pelham H R B. Sequence of a second human KDEL receptor. J. Mol. Biol. 1992;226:913-916.

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(6). Marasco WA, Haseltine WA, Chen S. Design, intracellular expression, and activity of a human anti-human immunodeficiency virus type 1 gp120 single-chain antibody. Proc. Natl. Acad. Sci, USA 1993;90:7889-7893.

(11). Appendices:

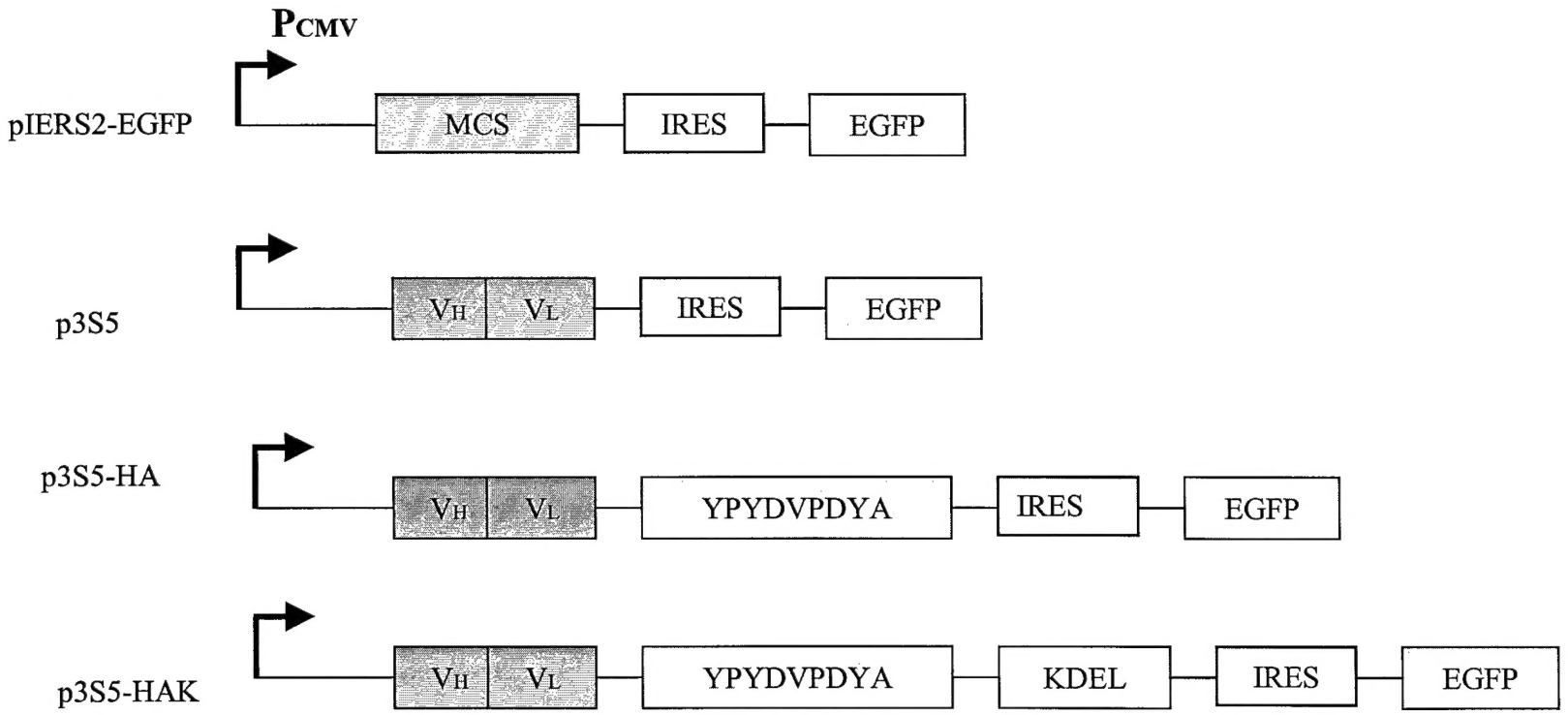
None.

(12). Final Reports:

One abstract has been accepted by the 4th Annual American Society of Gene Therapy Meeting (05/2001) and published on *Molecular Therapy* (05/2001).

One paper has been submitted to *Blood* (04/2001).

Principle investigator (Yurong Y. Wheeler) has received student payment from this award of $1, 292.01/month for the last one year.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MCS
IRES
EGFP
p3S5
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p3S5-HA
VH
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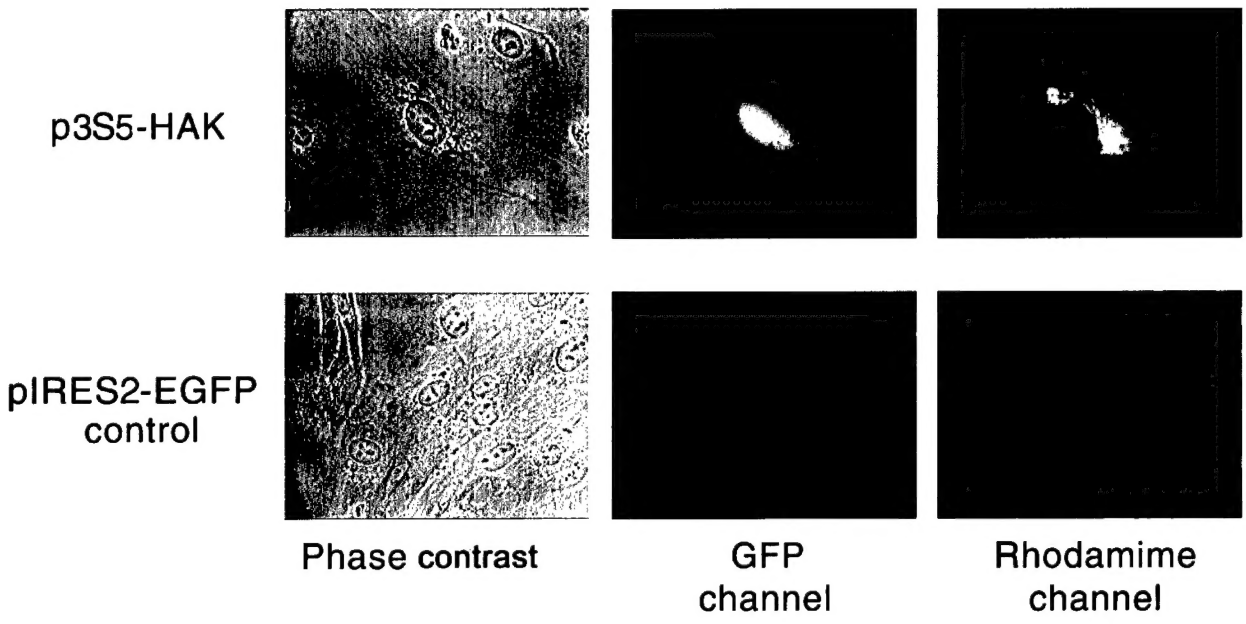
EGFP
p3S5-HAK
VH
VL
YPYDVPDYA
KDEL
IRES
EGFP

Fig. 1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control
Phase contrast
GFP
channel
Rhodamime
channel

Fig. 2

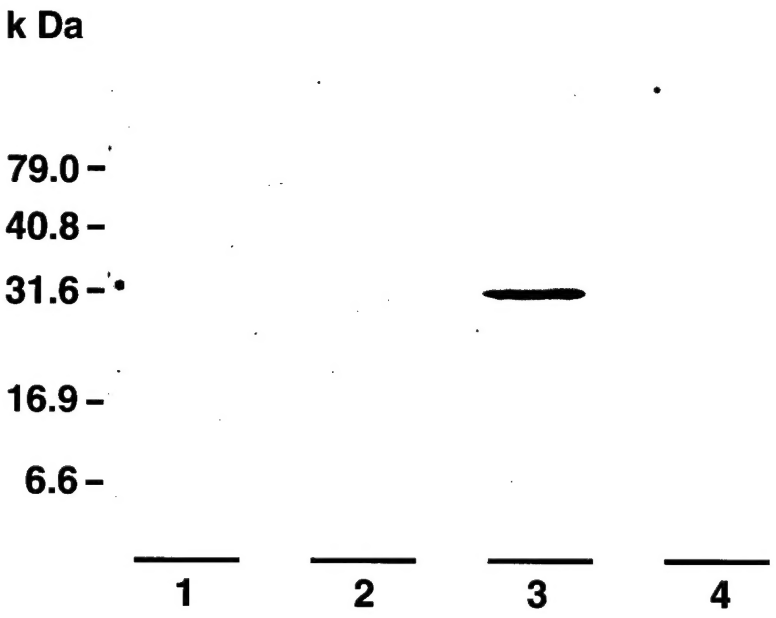
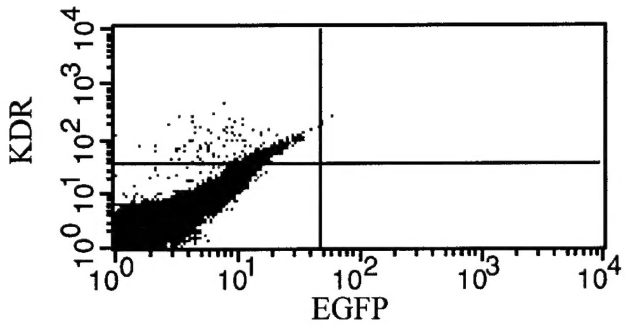


Fig.3

Fig. 4

A



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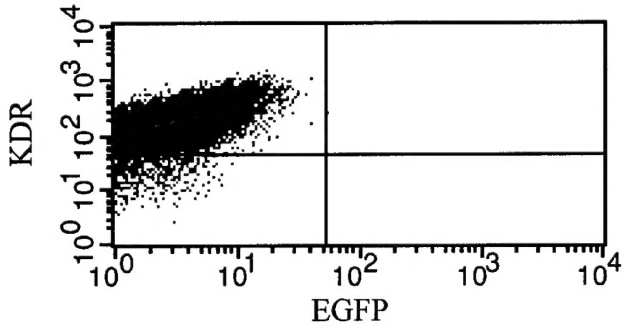
LL	97.14
LR	0.00

B



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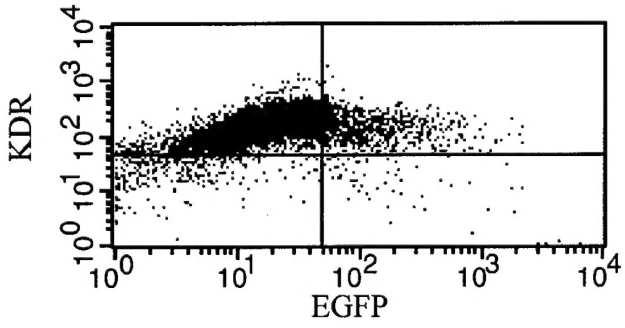
C



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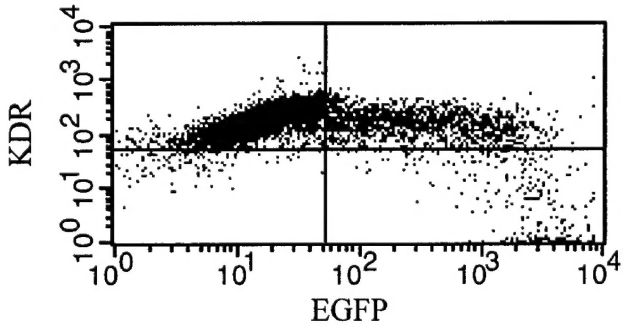
D



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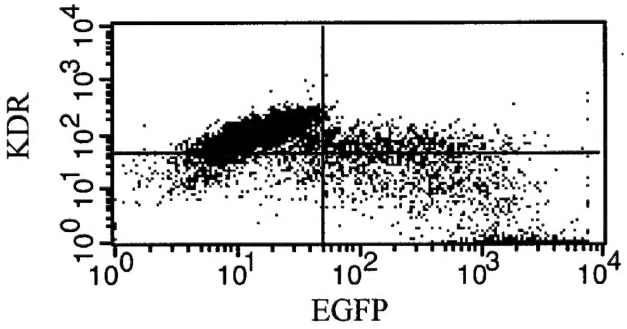
E



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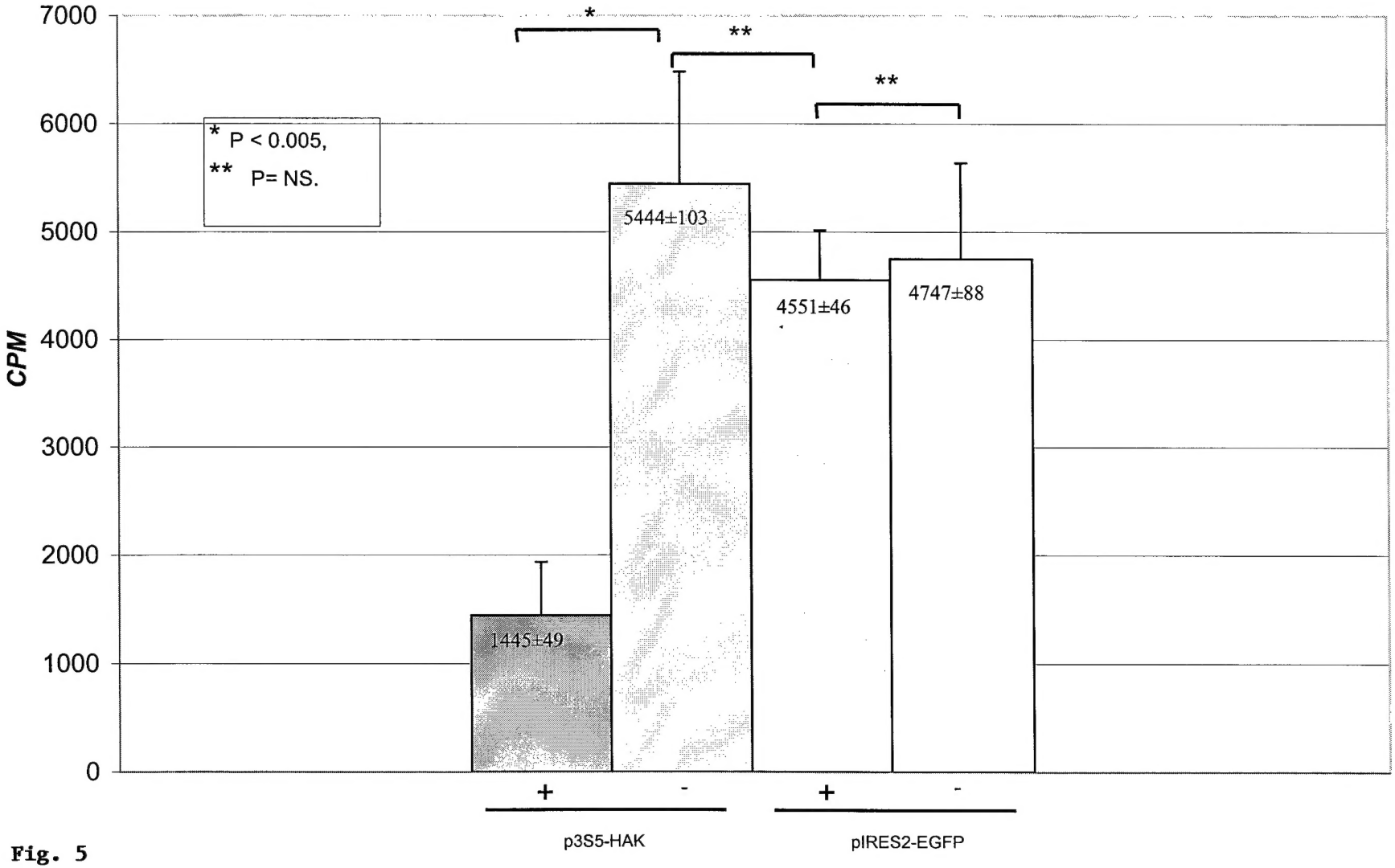
7000
6000
5000
4000
3000
2000
1000
0
CPM
* P < 0.005,
** P= NS.
*
**
**
1445±49
5444±103
4551±46
4747±88
+
-
+
-
p3S5-HAK
pIRES2-EGFP

Fig. 5

Table 1.

Analysis of flow cytometry assay of surface KDR expression on p3S5-HA, p3S5-HAK and control transfected HUVECs

Transfected plasmid	Transfection percentage	% KDR positive	
		all cells	transfected cells
pIRES2-EGFP	5.1 ± 2.2	92.5 ± 3.7	*(1)* 82.5 ± 12.5
p3S5-HA	9.8 ± 6.9	94.1 ± 2.3	*(2)* 78.6 ± 10.7
p3S5-HAK	13.3 ± 3.1	85.3 ± 1.1	*(3)* 27.9 ± 13.6

p-values (student t test):

(1) vs (3) $p < 0.01$

(1) vs (2) $p > 0.1$

(2) vs (3) $p < 0.01$